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VOLICITIN, AN ELICITOR OF MAIZE VOLATILES IN ORAL SECRETION OF *Spodoptera exigua*: ISOLATION AND BIOACTIVITY

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Abstract—Plants respond to insect-inflicted injury by systemically releasing relatively large amounts of several volatile compounds, mostly terpenoids and indole. As a result, the plants become highly attractive to natural enemies of the herbivorous insects. In maize, this systemic response can be induced by the uptake via the stem of an elicitor present in the oral secretions of caterpillars. Such an elicitor was isolated from the regurgitant of *Spodoptera exigua* larvae, identified as *N*-(17-hydroxylinolenoyl)-L-glutamine, and named volicitin. Here we present details on the procedure that was used to isolate volicitin and the biosasays that demonstrate its potency as an elicitor of maize volatiles that attract parasitoids. With a series of liquid chromatography purification steps, volicitin was separated from all other inactive substances in the regurgitant of larvae of the noctuid moth *S. exigua.* Maize seedlings that were incubated in very low concentrations of pure natural volicitin released relatively large amounts of terpenoids and became highly attractive to the parasitoid *Microplitis croceipes.* The identification of this and other insect-derived elicitors should allow us to determine their precise source and function, and better understand the evolutionary history of the phenomenon of herbivore-induced volatile emissions in plants.

Key Words—Volicitin, induced plant volatiles, elicitor, parasitoid attractants, *Zea mays*, *Spodoptera exigua*, *Microplitis croceipes.*

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INTRODUCTION

Feeding damage caused by herbivorous arthropods commonly results in chemical changes in plants that may serve several defensive functions (Baldwin, 1994). One function of herbivore-induced plant chemicals may be to attract natural enemies of the herbivores. This is indicated by studies on the volatile releases induced by spider mites in a variety of plants (Dicke and Sabelis, 1988; Dicke et al., 1990) and caterpillars (Turlings et al., 1990; Steinberg et al., 1993). These studies demonstrate an active emission of specific blends of volatiles (mostly terpenoids) throughout the herbivore-injured plants, even from undamaged leaves (Turlings and Tumlinson, 1992; Röse et al., 1996). The emitted volatiles are exploited by predatory mites and parasitoids that are in search of prey and hosts, respectively.

In maize the release of volatiles can be triggered by applying regurgitant from caterpillars on mechanically damaged sites (Turlings et al., 1990; Potting et al., 1995). Mechanical damage by itself results in releases of only small amounts of volatiles, while application of regurgitant on undamaged leaves triggers no response. However, volatile releases can be induced in undamaged leaves by incubating seedlings with their cut stem in a solution that contains regurgitant (Turlings et al., 1993). Apparently, an elicitor in the regurgitant can trigger the emission of volatiles by the leaves even if the surface of the leaves is left unharmed. The volatile releases correspond to a highly significant increase in attractiveness of the plant to parasitic wasps (Turlings et al., 1993). Other insect-derived materials, such as feces and hemolymph, evoked only a marginal response in plants. Furthermore, eliciting factors in the regurgitant appear not to be directly derived from the food that the insects eat and occur in the regurgitant of all of five lepidopterous species tested as well as in the regurgitant of grasshoppers (Turlings et al., 1993, but see Paré et al., 1998). Compared to other species, regurgitant collected from the beet armyworm, *Spodotera exigua*, is particularly active (see also Figure 1) and was therefore chosen for isolation of the elicitor.

Cabbage plants too will release volatiles that are attractive to parasitoids after they have been damaged and the damaged sites are treated with regurgitant of *Pieris brassicae* larvae (Sato, 1979; Mattiacci et al., 1994). β-Glucosidase is suspected to be the main factor in *P. brassicae* regurgitant that elicits this response in cabbage. Treating cabbage plants with β-glucosidase results in the emission of volatiles similar to those emitted following caterpillar attack, and the treated plants become attractive to *Cotesia glomerata*, a parasitoid that attacks *P. brassicae* (Mattiacci et al., 1995).

In the case of induced maize volatiles, the insect elicitors are not enzymes, but rather fatty acid-derived molecules that appear to interfere with the octadecanoid signaling pathways in higher plants. The elicitor from *Spodoptera* oral secretion was recently identified as *N*-[17-hydroxylinolenoyl]-L-glutamine and

was named volicitin (Alborn et al., 1997, 2000). In the current study, we describe all details of the isolation process and the bioassays that were conducted to demonstrate its activity, both in terms of eliciting volatile emissions as well as the behavioral responses that the emissions evoke in a parasitic wasp.

METHODS AND MATERIALS

Collection of Caterpillar Regurgitant. Third instars of the beet armyworm, *Spodoptera exigua*, were obtained from rearing facilities at the USDA-ARS (Gainesville, Florida). They were placed on corn seedlings for 12–16 hr before their regurgitant was collected. The collection procedure has been described by Turlings et al. (1993). Briefly, well-fed caterpillars were held with a pair of lightweight forceps and gently squeezed in the head region with another pair. This caused the caterpillars to empty their foregut content (regurgitant), which was collected by drawing it via a 100-μl capillary tube into a vial under low vacuum.

Incubation of Maize Seedlings. Maize (*Zea mays* L.) seedlings of the variety Ioana sweetcorn were grown in metal trays ($9 \times 35 \times 50$ cm) in a greenhouse. Approximately 60 seeds were planted in a 50 : 50 mixture of moist vermiculite and potting soil. From 6 AM to 8 PM natural light was supplemented with 400-W high-pressure sodium lamps placed 1 m above the trays. Seedlings were used in experiments when they carried three leaves, 8–10 days after planting. They were harvested from the trays by cutting their stems close to the soil at 9–11 PM. Immediately afterwards, the cut end of each seedling was placed in a 500-μl test solution (see below) in a 1-ml vial. Usually, six solutions were tested with three seedlings per solution. The following day the plants were removed from their vials at 9–10 AM, the submerged part of the stems was cut off, and the severed ends were wrapped in wet cotton wool. Seedlings that had been submerged in the same test solution were kept together and either placed in a chamber of the volatile collection apparatus or used in a wind-tunnel bioassay to determine their attractiveness to a parasitoid.

Collection and Analysis of Volatiles. Volatiles from each group of three seedlings were collected under artificial light in an apparatus that was described by Turlings et al. (1991b). The technique allows for the collection of six groups of seedlings in parallel glass chambers. Pure, humidified air was blown over the plants and pulled out at a rate of 600 ml/min through a trap containing 25 mg of Super Q adsorbent (Alltech Assoc., Deerfield, Illinois). After a collection of 2 hr, the traps were removed and extracted with 150 μl of methylene chloride. One or two internal standards (*n*-octane and *n*-nonyl-acetate, 20 ng/μl) in 50 μl of methylene chloride were added. Of the extracts, 2.5 μl was injected with a Hewlett Packard autoinjector (model 7673) in on-column mode onto a Quadrex methyl silicone column (50 m $\times$ 0.25 mm ID, 0.25-μm film) preceded by a 10-m

× 0.25-mm-ID deactivated retention gap inside a Hewlett Packard model 5890 gas chromatograph. The temperature was programmed from 50°C, at 5°C/min, to 180°C. Chromatographic data were processed with Turbochrom 3.1 software (Perkin-Elmer, Norwalk, Connecticut) and relative quantities for each compound were calculated based on the peak areas of the internal standard. The identities of the eight most relevant compounds were determined in a previous study (Turlings et al., 1991b).

Isolation Procedure. Details on the various purification steps are given below. Each step usually started with a pilot separation with 0.5 ml regurgitant, followed by a scaled-up process with up to 50 ml of regurgitant. For the first clean-up, regurgitant was rinsed through solid-phase extraction (SPE) cartridges (Bakerbond; J. T. Baker, Philipsburg, New Jersey). Further isolation of the active component was accomplished by three subsequent reverse-phase HPLC analyses and simultaneous collection of fractions, by using a LDC 4100 pump with a SM5000 diode array UV detector (LDC Analytical, Riviera Beach, Florida), and monitoring wavelengths from 190 to 360 nm. For each analysis, a combination of water and acetonitrile was pumped through the column at 1 ml/min. After injection of active material, 1-min fractions were collected. Each fraction was then concentrated to dryness under vacuum (Speed Vac rotary concentrator; Savant Instruments, Farmingdale, New York) and redissolved to its original volume with 50 mM Na_2HPO_4 buffer (titrated to pH 8 with 1 M citric acid). To test if fractions contained active material, an amount equivalent to 25 μl of the original regurgitant was added to 500 μl of the same buffer, and maize seedlings were incubated in the solutions and the volatiles released were measured as described above. Seedlings that were incubated in a solution with 25 μl of "crude" regurgitant, and seedlings incubated in buffer alone served as positive and negative controls, respectively. In each case, a combination of all fractions was also tested to ensure that no activity was lost by leaving out certain fractions. Only those fractions that elicited the release of terpenoids in the seedlings were used for further isolation steps.

Flight-Tunnel Tests with Parasitic Wasps. Flight-tunnel bioassays were used to determine the attractiveness to the parasitoid *Microplitis croceipes* of maize seedlings that had been incubated in various solutions. The wasps were obtained from the USDA-ARS, Insect Biology and Population Management Research Laboratory, Tifton, Georgia. They were reared according to the procedure described by Lewis and Burton (1970) and shipped to the laboratory in Gainesville as cocoons. Flight-tunnel tests were conducted with 3- to 5-day-old females. Just prior to their release in the tunnel, the wasps were allowed to oviposit in a single *Helicoverpa zea* larva that was feeding on a maize seedling. Larvae had been placed on seedlings 14–18 hr before. This experience increases the wasps' responsiveness significantly (Drost et al., 1986; Eller et al., 1988).

The flight-tunnel described by Turlings et al. (1991a) was used. The conditions inside the tunnel were 15 cm/sec airflow, 55–70% relative humidity,

27–29°C, and approx. 500 lux light. Upwind inside the tunnel, two groups of three seedlings were placed in separate vials on a 40-cm-high stand 20 cm apart. Each vial contained seedlings that had been incubated for at least 12 hr in a different solution. For a particular combination of seedlings, two wasps were released in the tunnel 80 cm downwind from the seedlings. The plant on which they landed was recorded, after which the relative positions of the seedlings were switched, and two new females were tested. After four wasps were tested, a new combination of seedlings was placed in the tunnel. Each treatment combination was repeated six times. For each combination of odor sources, we tested 24 females and a binomial test was used to determine significant preferences for a particular odor source ($\alpha = 0.05$).

A wind-tunnel test was conducted to demonstrate the increased attractiveness of seedlings that had been incubated in crude regurgitant as compared to seedlings that had been placed in distilled water. In a later wind tunnel test, plants that had been incubated in the isolated elicitor were compared with plants that had been placed in "crude" regurgitant.

RESULTS

Effect of Caterpillar Regurgitant on Volatile Emissions and Attractiveness of Maize Seedlings. To evaluate the bioassay and to confirm the effects of caterpillar regurgitant, seedlings were placed in either 500 μl distilled water (control) or 25 μl regurgitant diluted with 475 μl distilled water as described above. Figure 1 shows that plants treated with regurgitant produced large amounts of terpenoids, while the plants that had been standing in water emitted very little. This corresponded well with the attractiveness of the plants; of 24 wasps tested, 20 flew to the treated plants, 2 to the control plants, and 2 did not make a choice. The preference was highly significant (binomial test, $P < 0.001$).

Acidification and Filtering of Regurgitant. In a first clean-up, crude regurgitant was centrifuged at 16,000*g* for 30 min to eliminate solid material, and the supernatant was filtered through a 0.22-μm sterilizing membrane (Millex GV, Millipore, Bedford, Massachusetts). Next, 1 ml of 0.2 M Na_2PO_4/citric acid in water adjusted to pH 3.3 with 1 M citric acid was added to 1 ml of filtered regurgitant. This resulted in the coagulation of proteins in the solution. The proteins were precipitated by centrifuging for 30 min at 16,000*g*, and again the supernatant was filtered through a 0.22-μm membrane. The filtered regurgitant was highly active and therefore the equivalent of only 25 μl solution was used in subsequent bioassays.

Solid-Phase Extraction. For the first isolation step, a 6-ml C_{18} bonded reverse-phase (Bakerbond SPE octadecyl) solid-phase extraction (SPE) column was used. The column was conditioned by rinsing with 10 ml CH_3CN followed

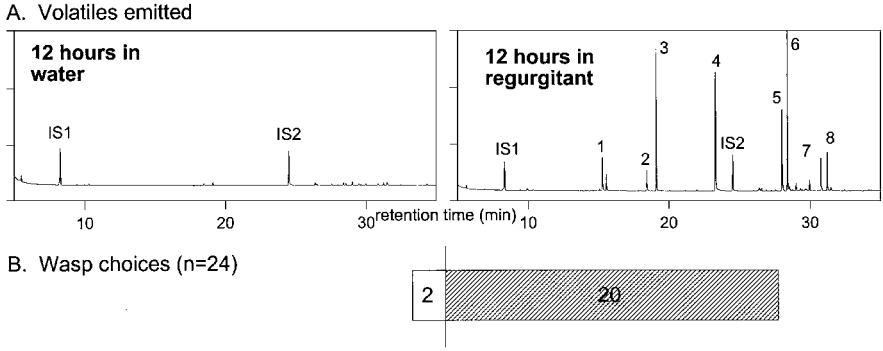

FIG. 1. (A) Chromatographic profiles of volatiles collected from corn seedlings that had been incubated for 12 hr in either distilled water or in a solution with 50 μl caterpillar regurgitant. The identities of the various compounds are 1, (*Z*)-3-hexen-1-yl acetate; 2, linalool; 3, (3*E*)-4,8-dimethyl-1,3,7-nonatriene; 4, indole; 5, α-*trans*-bergamotene; 6, (*E*)-β-farnesene; 7, (*E*)-nerolidol; and 8, (3*E*,7*E*)-4,8-12-trimethyl-1,3,7,11-tridecatetrane; IS1, internal standard, *n*-octane; IS2, internal standard, nonyl acetate. (B) Graphic depiction of the numbers of *Microplitis croceipes* females landing on maize seedlings in a wind tunnel when they were offered a choice between seedlings incubated in water and seedlings incubated in a regurgitant solution. The females showed a significant preference (binomial test) for regurgitant-treated plants. Of the 24 females tested, only two did not make a choice.

by 10 ml of water. Aliquots (0.5 ml) of filtered material were placed on the conditional column and three fractions were collected by rinsing the column with 2 ml of water, 2 ml of 50% CH_3CN in water, and 2 ml of 100% CH_3CN, respectively. The three fractions were concentrated to dryness and redissolved in 0.5 ml of 50 mM Na_2HPO_4 adjusted to pH8 with 1 M citric acid. Seedlings were incubated in 500 μl of the same buffer containing one of the fractions or a recombination of all fractions at concentrations equivalent to 25 μl of regurgitant. All activity was retained in the 50% CH_3CN fraction (Figure 2). This fraction was used for the subsequent step.

Fractionations by HPLC. For the first HPLC fractionation, 50-μl aliquots of the 50% CH_3CN SPE cartridge fraction were injected onto a Waters Nova Pac C_{18} reverse-phase column (4 μm, 4 mm ID $\times$ 150 mm column; Waters, Millford, Massachusetts). The samples were eluted with a solvent gradient of 0 to 25% CH_3CN in water in 15 min, followed by an increase to 100% acetronitrile in the next 15 min, with a flow of 1 ml/min. All material appeared to elute during the first 15 min (Figure 3). Only the plants that were incubated in the 8 + 9-min fraction showed a strong increase in volatile emissions (Figure 3).

The 8 + 9-min fraction was further fractionated on a YMC 18 ODS-AQ S-5

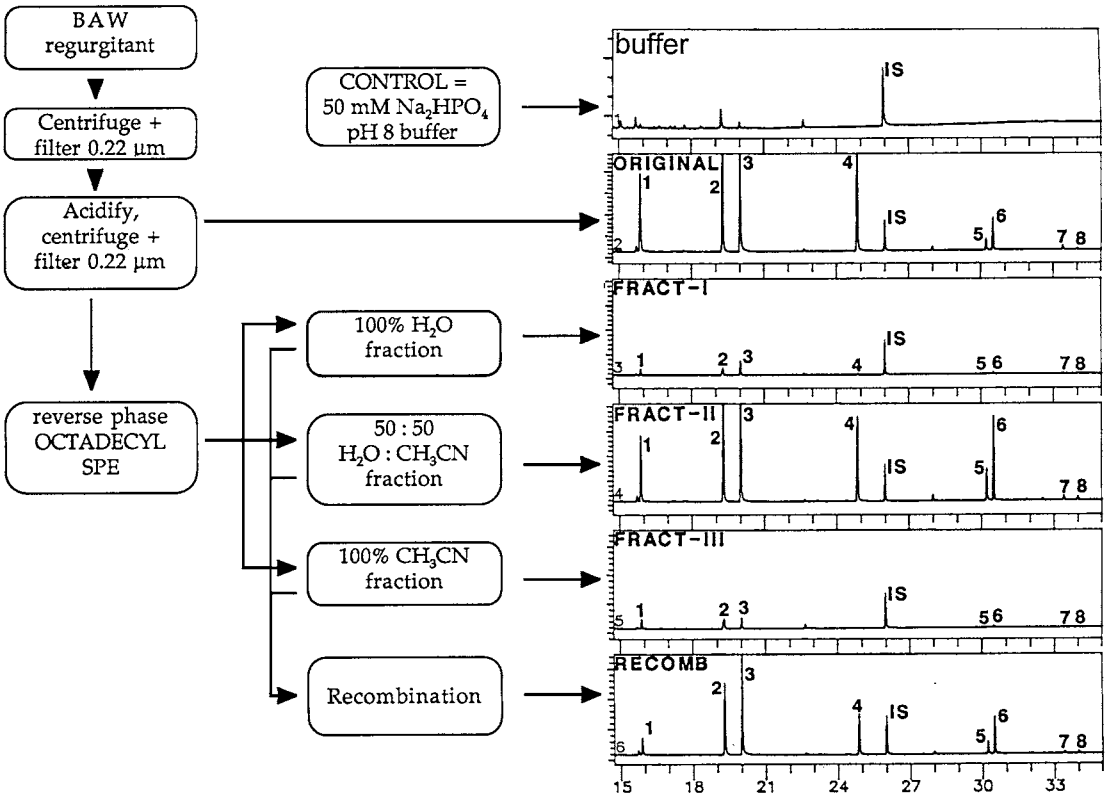

FIG. 2. First purification scheme using a 6-ml C_{18} bonded reverse-phase solid-phase extraction column. Crude regurgitatnt was eluted from the column with water, 50% CH_3CN in water, and 100% CH_3CN, respectively. The chromatograms represent the odors emitted by corn seedlings incubated in the different fractions and various controls. The 50% CH_3CN retained all activity (for peak identities see Figure 1).

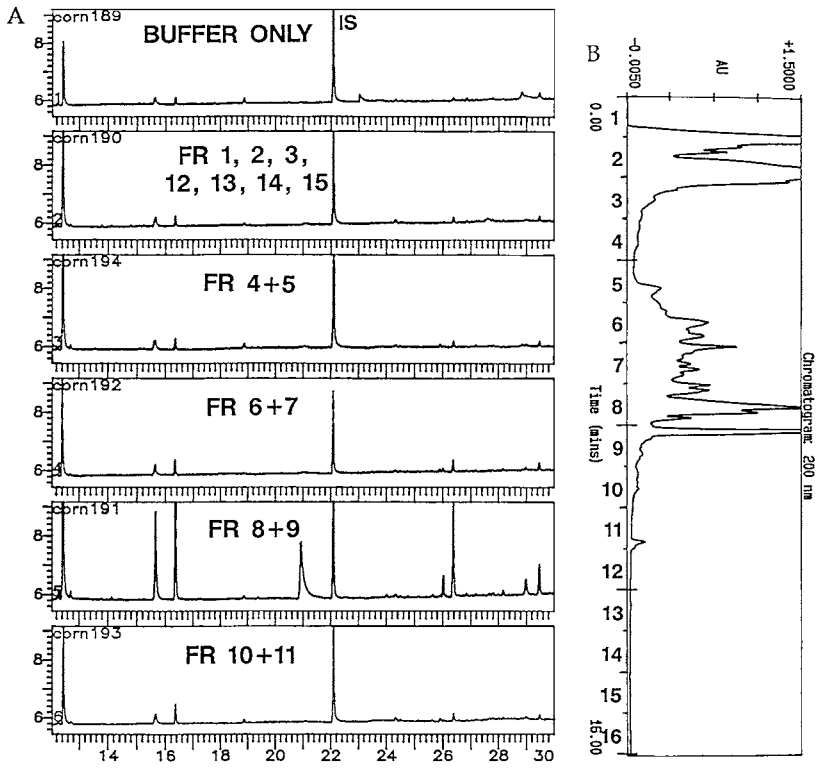

FIG. 3. Activity of fractions collected during an HPLC analysis on a C_{18} reverse-phase column (4 μm, 4 mm ID $\times$ 150 mm column). Samples were eluted with a solvent gradient of 0 to 25% CH_3CN in water in 15 min, followed by an increase to 100% acetonitrile in the next 15 min, using a flow of 1 ml/min. The chromatograms (A) of plants incubated in various fractions showed that all activity was retained in fractions 8 + 9. The HPLC chromatogram revealed several large peaks in these fractions (B).

reverse-phase column (200 Å, 46 mm ID $\times$ 250 mm; YMC Co., Ltd., Kyoto, Japan) by injecting amounts equivalent to 1 ml of the original regurgitant. The same solvent gradient as above was used (Figure 4A). One-minute fractions were collected, prepared, and bioassayed as above. All active material was collected in fractions 21–23 (Figure 4B).

The two overlapping peaks in the 21–23 fraction could be separated to baseline on the same column with a solvent gradient of 20–60% acetonitrile in water over 20 min. The resulting chromatogram (Figure 5) showed three peaks. All activity was retained in the fraction with the largest peak. Plants that were incubated in a combination of all fractions before this peak or in a combination of

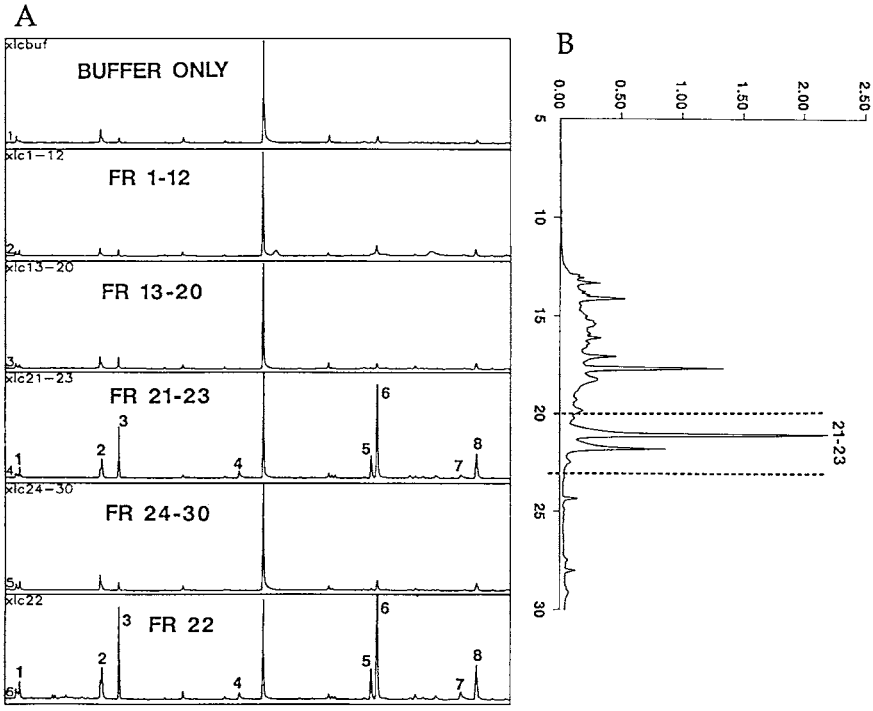

FIG. 4. HPLC analysis (B) of the active fractions (8 + 9) from the previous step (Figure 3). Chromatographic profiles (A) of odors collected from seedlings incubated in the different fractions revealed that fractions 21–23 retained all activity (for peak identities see Figure 1). Two large peaks were collected with these fractions (B).

all fractions after this peak emitted no detectable amounts of volatiles. Plants that were incubated in the fraction with this peak, emitted large amounts of the induced volatiles (Figure 5).

Effects of Isolated Elicitor on Attractiveness of Maize Seedlings. Using the same flight tunnel bioassay as described above, we tested whether plants that were incubated in a solution with an amount of isolated elicitor equivalent to 25 μl regurgitant were as attractive to parasitoids as plants incubated in a regurgitant solution. Three treatments were used: (1) 500 μl of buffer only, (2) 25 μl of filtered regurgitant in 500 μl buffer, and (3) 25 μl regurgitant equivalent of isolated elicitor in 500 μl buffer. Individual seedlings were placed in one of the three solutions for a period of 12 hr and subsequently used in two-choice tests in the flight tunnel. With each of the three possible combinations of the seedlings, 24 experienced *Microplitis croceipes* females were tested.

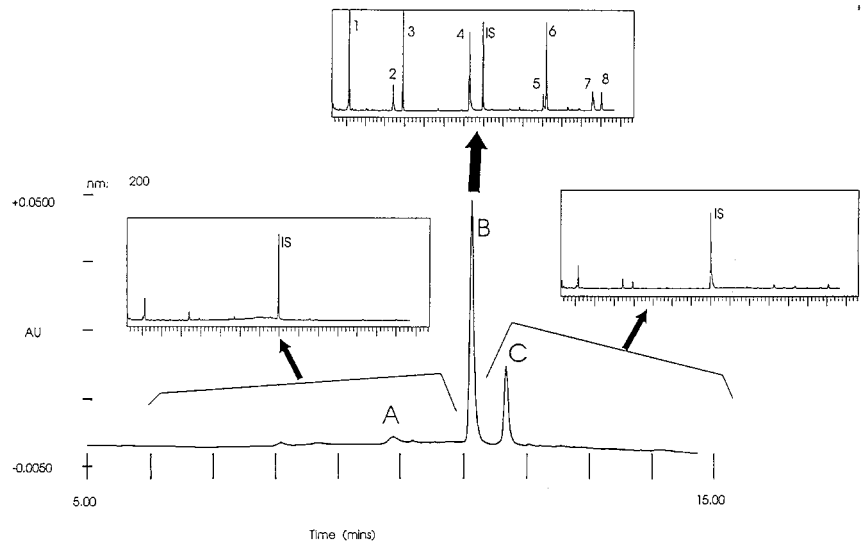

FIG. 5. Further purification of active fractions 21–23 (Figure 4) on the same YMC 18 ODS-AQ S-5 reverse-phase column as used in the last step. The chromatograms show that only seedlings that were placed in a solution that contained peak B emitted the inducable volatiles (for peak identities see Figure 1).

The results, summarized in Figure 6, show that plants that had been placed in either regurgitant (binomial test, $P < 0.001$) or elicitor (binomial test, $P < 0.001$) were strongly preferred over plants that had been placed in buffer only. Furthermore, when offered a choice between plants treated with regurgitant and plants treated with the elicitor, the wasps showed no preference (binomial test, $P = 0.678$).

Final Purification. During the HPLC purifications, the retention time of the active material varied with the pH of the solution. It increased at low pH and decreased at high pH. This indicated that the active component contained a weakly acidic functional group, which was confirmed by a complete extraction of the active material into methylene chloride from pH 3 solution. All activity remained in the aqueous phase when a solution at pH 8 was extracted with methylene chloride. All activity could be extracted back into pH 8 buffer from the organic phase.

A methylene chloride solution of the active material was further fractionated on a 3-ml 10SPE diol cartridge (Bakerbond) which first had been activated with a rinse of methanol and methylene chloride. A 5-ml sample was placed on a diol cartridge and eluted with 3 ml methylene chloride followed by 2 ml of methanol. All activity was found to be in the methanol fraction. Rechromatography of this

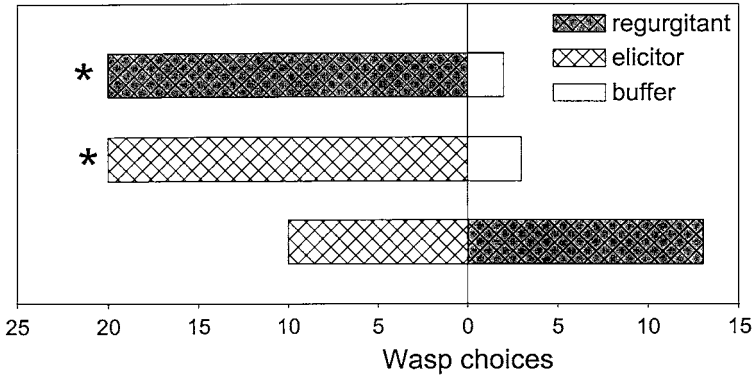

FIG. 6. Responses of *Microplitis croceipes* females during two-choice flight tunnel tests to corn seedlings. The bars indicate the total number of wasps (N = 24) that flew to a particular type of seedling. The asterisks indicate statistically significant preferences for a particular treatment (binomial test). The wasps did not distinguish between seedlings that had been incubated in crude regurgitant and those that had been incubated in an equivalent amount of the isolated elicitor. Both treatments were strongly attractive to the wasps.

fraction on the YMC HPLC column indicated that it was 99% pure. This material was used for the identification of the active component (Alborn et al., 1997).

DISCUSSION

A single compound, *N*-(17-hydroxylinolenoyl)-L-glutamine (volicitin), was isolated from caterpillar regurgitant that was as active as the original regurgitant in inducing volatile releases in corn seedlings. No other fraction obtained during the isolation process showed biological activity. The parasitoid *Microplitis croceipes* was strongly attracted to maize seedlings that had been incubated in the purified elicitor. In flight-tunnel bioassays the wasps were equally attracted to seedlings that had been incubated in regurgitant as compared to seedlings that had been incubated in an amount of isolated volicitin equivalent to what is found in regurgitant. Therefore, volicitin appears to be responsible for the induced release of volatiles.

Flying to plants treated with BAW regurgitant or volicitin constitutes a "mistake" for *M. croceipes* because it cannot use BAW as a host. As it and other parasitoids will readily make such mistakes in the laboratory (Turlings et al., 1993), we had argued that the plants respond the same to feeding by different herbivores. Recent evidence, however, shows that some parasitoids are able to distinguish the odors emitted from plants under attack by their specific

hosts from odors emitted by plants after attack by a nonhost (De Moraes et al., 1998). It is possible that different herbivores produce different elicitors or different blends of the same type of elicitors, thus causing the plants to emit different odors. Recently, we isolated and identified several volicitin-related compounds from BAW regurgitant (Alborn et al., 2000). None of these induce a comparable reaction in corn at natural concentrations, but at least one of them, *N*-linoleoyl-L-glutamine causes a much weaker reaction (about 30% on mol/mol comparison; Alborn unpublished data). Perhaps the contributions of other, less active elicitors cause slight differences in volatile emissions induced by different herbivores. These differences might be used by parasitoids to find their specific hosts, if not innately, perhaps through experience and associative learning.

Although much remains to be answered on the bioactivity of volicitin and related compounds, it appears that it interacts with the octadecanoid signaling pathway. This notion is strengthened by the fact that the inactive component **C** in Figure 5 was identified as *N*-(17-hydroxylinoleoyl)-L-glutamine (Alborn et al., 2000), which is derived from linoleic acid rather than linolenic acid as is the case for volicitin. Of these two unsaturated fatty acids, only linolenic acid is a precursor in the biosynthetic pathway leading to jasmonic acid, a key component in systemic plant wound responses.

The source of the elicitor within the caterpillar is still unknown. To come in contact with plant tissue, it should be present in the caterpillars' saliva. Therefore glands near the mouthparts are likely sources. Our experiments do not reveal how much, if any, of the elicitor is introduced to the plant during the feeding process. We can assume that it comes in contact with the plant tissue because while caterpillar feeding causes a strong reaction, mechanical damage alone causes an insignificant response (Turlings et al., 1990; Paré and Tumlinson, 1997). Plants could also respond to insect enzymes that transform plant cell membrane fatty acids to glutaminated hydroxy acids. This could be the case for another insect-derived elicitor that was studied by Mattiacci et al. (1995). They showed that in cabbage plants β-glucosidase induces the emission of glucosinolates, which are attractive to the parasitoid *Cotesia glomerata.* However, β-glucosidases are usually concentrated in the midgut of insects (Yu, 1989). It is unlikely that they play an important role as elicitors of the volatile emissions in maize. Further research on the source of the elicitor and the feeding behavior of caterpillars is needed to fully understand the specific interactions.

Another critical question of ecological and evolutionary importance is why caterpillars produce a compound that elicits a reaction in plants that can be detrimental to the caterpillars? At this point we can only speculate. These elicitor compounds may serve essential functions for the caterpillar, perhaps in the digestion of plant material. They might also help the insect to absorb essential fatty acids. Interestingly, linolenic acid is the precursor for the so-called "green leaf volatiles" (Paré and Tumlinson, 1996). These C_6 alcohols, aldehydes, and

acetates can be toxic to insects. Paré et al. (1998) showed that upon consumption by caterpillars, plant-derived linolenic acid is converted to the 17-hydroxy compound and coupled with the glutamine moiety to form volicitin. Perhaps this mechanism simply provides a way to pull the fatty acid out of the system before lipoxygenase activity in the damaged plant tissue transforms them to the noxious green leaf volatiles. Volicitin provides us with a chemical tool with which a variety of these remaining questions on the biology and physiology of plants and insects might be answered.

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